

PLASMA SULFATE ANALYSIS
I. Benzidine Method

by

Lewis M. Fetterman Jr.

Senior Thesis
May 1972

A C K N O W L E D G M E N T

The author is indebted to the entire Washington and Lee Chemistry Department especially Dr. J. Brown Goehring, Dr. James K. Shillington, and Dr. John H. Wise for their generous help during the past 4 years. I am deeply indebted to Dr. Esmarck S. Gilreath for his excellent introduction to analytical chemistry and guidance.

Finally the author is most deeply indebted to Dr. Michael Pleva for his invaluable guidance, advice, and assistance not only in the preparation of this thesis but also in many other areas to numerous to list.

Lewis M. Fetterman, Jr.
Lexington, Virginia
May, 1972

PLASMA SULFATE ANALYSIS
I. Benzidine Method

The quantitative determination of micro and ultra micro amounts of sulfate has historically been a problem to analytical chemists. The determination is further complicated if the analysis is to be performed on a complex solution where only a small amount is available. This is the situation one faces when attempting to analyze the quantity of sulfate in 50-200 μ l. of blood plasma. A literature search was made in order to evaluate the available methods, and to attempt to find an analysis that would work in the adverse conditions of blood plasma. For a detailed list of analysis methods, the reader is referred to Kolthoff and Elving's Treatise on Analytical Chemistry.

The oldest methods for the analysis of sulfate involve the precipitation of the barium sulfate salt. Originally this analysis was carried out by weighing the precipitate. Later developments allow analysis by titrimetric methods using complexing indicators for the detection of excess barium ions. Turbidimetric, and EDTA complexometric methods are also now available. In addition a few colorimetric indicators have been developed. There are two major causes of error in these methods. The first error producing factor is the slight solubility of barium sulfate. A second cause of error is the other ions that are absorbed by the crystal. For full scale analysis in relatively small interfering ion concentrations, these two factors tend to cancel each other out. But, for micro and ultramicro analysis of small samples with high interfering ion concentration, these two problems become of major im-

portance. Investigations made by the Washington and Lee Biology Department,¹ indicated the inability of barium precipitation methods in analysis of small quantities of plasma, even if radioactive methods² are used for detection of the small amounts of barium used. As a result of their findings, i.e. difficulties with the method, it was decided to attempt an adaption of a method other than barium precipitation methods.

Other commonly used methods to quantitatively analysis for sulfate involve the use of lead ions as a precipitating reagent. The analysis methods that have been developed for lead sulfate are similar to those used in connection with barium. In addition amperometric methods are available for both end point detection and concentration measurement. Since these methods involve the same interferences found in the barium methods, it was felt in light of the failure of the barium technique, that an attempt to use a lead method would prove fruitless. Therefore our attention was turned to the other two types of sulfate analysis.

One of these types, the conversion methods, includes the analysis of sulfate by conversion to sulfur dioxide³ or hydrogen sulfide⁴ followed by a gas analysis procedure. These methods seem to be most sensitive and very applicable since the conversion of the sulfate to a gas will remove the sulfate from the interferences contained in the plasma. But since it was desired to avoid the handling of gases if possible, these methods were not tried in the hope that another method could be found.

The other methods available for sulfate analysis involve

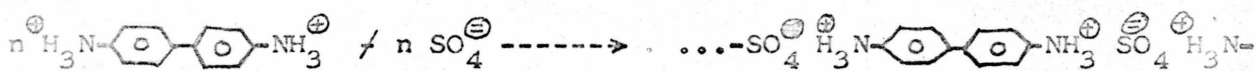
the use of a large organic ion as a precipitating reagent. Using organic ions it is possible to produce more insoluble salts and use other detection methods. The most attractive detection method is UV spectrophotometry. E.C. Owen in 1936 suggested the use of benzidine as a precipitating reagent. The reagent worked well but suffered phosphate interference. R. Belcher and his colleagues have suggested many other organic reagents that are more insoluble than benzidine including 4-chloro-4' aminodiphenyl⁵ and octoamino μ -amino- μ -nitrodicobalt-III nitrate.⁶ The 4-chloro-4' amino diphenyl (CAD) is considered to be one of the best reagents available today. This reagent can be titrated with Sodium Hydroxide or measured turbidimetrically.⁷

The most attractive method found was that of Lars Andersen of the University of Helsingfors, Finland.⁸ Andersen suggests analysing for sulfate by precipitating it with benzidine, washing the precipitate thoroughly, redissolving it in acid solution, and measuring the ultraviolet absorption of the solution. Anderson reported result with an accuracy of 1-2%. He also points out that as reported earlier phosphate interferes with the analysis. Consult his paper for a more detailed discussion of the method. It is the aim of this project to re-test the reliability of the method and then attempt to apply it to small amounts of plasma samples. This method was selected since Andersen suggests an ultramicro technique which could possibly detect the small amount of sulfate present in 100 μ l. plasma samples (the proposed test volume). The method seems quite appropriate since it involves

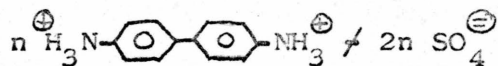
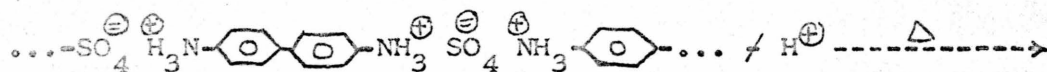
precipitation of the sulfate that would remove it from the interferences found in the plasma and make UV detection possible. The method was to be tested on the micro level, and then if it worked, on the ultramicro level. Since phosphates and possibly proteins would interfere with this test, the phosphate-protein removal by uranium acetate was used. This method was suggested by E. Miller, ⁹ et. al. for use with plasma samples. Miller's tests seem to indicate the method is good for removing phosphate and proteins but not sulfate. In order to limit the dilution of the blood 2/3 ml. of 24 g./l. uranium acetate was used for each 1 ml. of plasma rather than the 2 ml. of 8 g./l. uranium acetate they suggest. For further discussion of this method consult their paper.

Reactions.

Precipitation:



Dissolving:



EXPERIMENTAL

Apparatus. 1) Bechman DB Spectrophotometer with UV source; 2) two 2ml. pipets and one 100 ml. pipet; 3) four 25 ml. glass stoppered erlenmeyer flasks; 4) one (or more) 1.5 cm. Büchner funnel; 5) No. 42 (or slow) filter paper; 6) six 1 liter glass stoppered storage bottles; 7) four 1 liter volumetric flasks; 8) Wash Bottle; 9) 4 oz. bottle with dropper; 10) 4 liter storage bottle; and 11) four 400 ml. beakers.

Reagents. 1) 6.7 g. Benzidine; 2) 20 ml. of 6N HCl; 3) Glacial Acetic Acid; 4) Ethanol; 5) Hydrochloric Acid; 6) Distilled Water; and, 7) uranium acetate.

Reagent Preparation. 1) Benzidine hydrochloride. Dissolve the 6.7 g. Benzidine in the 20 ml. 6N HCl and dilute to 1 liter. After allowing to stand over night, filter and store in labeled 1 liter storage bottle. After lengthy storage mold will grow and the solution should be discarded. 2) 0.2N Acetic Acid. Dilute 11.4 ml. of glacial Acetic Acid to 1 liter and store in labeled 1 liter storage bottle. 3) 50 % Ethanol. To 125 ml. of Ethanol add 125 ml. of distilled water and store in the 4 oz. bottle. 4) Dilute HCl. Prepare 4 l. by adding 1 l. of Hydrochloric Acid to 3 l. of distilled water. 5) Uranium Acetate. Dissolve 24g. in 1 liter of distilled water.

Sample Preparation. 1) To a sample of 1 to 10 ml. containing from .1 to 1.5 mg. of sulfate in a 25 ml. Erlenmeyer flask, is added 2 ml. of 0.2N Acetic Acid. After mixing this solution briefly, 2 ml. of Benzidine hydrochloride reagent is added and

the solution allowed to stand for at least 10 minutes. After the precipitation has completed, the solution is filtered under suction through the Büchner funnel. The Erlenmeyer flask and precipitate are washed with a few ml. of 50 % ethanol. Then the whole funnel is placed in 200 ml. of warm dilute HCl (pipetted) contained in a 400 ml. beaker. After the benzidine sulfate precipitate has dissolved, the funnel and filter paper are removed and rinsed thoroughly with distilled water. This acid solution is then transferred to a 1 liter volumetric flask and the beaker rinsed thoroughly with distilled water. The rinse water added to the solution. After the solution has cooled, it is diluted to 1 liter and well mixed. The apparatus described allows up to 4 samples to be prepared before measuring on the spectrophotometer. It is suggested that 3 or 4 samples be prepared at the time, since it takes approximately 1.5 hours to prepare one sample and only about two hours to prepare 4 together, once the procedure is well organized. (These times exclude any sample preparation other than measuring out sample volume). The well-mixed samples are then run on the DB at 2500 Å using HCl Reference Solution as a reference. The samples should be run twice each along with the standards (also should be run twice) in a completely random order.

Standard Preparation. Standards for running with the samples can be prepared by dissolving 515.3 mg. of Ammonium Sulfate in 250 ml. of distilled water and preparing benzidine standards by treating 1, 2 and 3 ml. samples as above. This will produce .5

mg. $SO_4^{=}$, 1.0 mg. $SO_4^{=}$, and 1.5 mg. $SO_4^{=}$ standard which should be stored in storage bottles for use during each measurement. The HCl Reference solution is prepared by diluting 200 ml. of the Dilute HCl solution, prepared earlier to 1 liter. When the Dilute HCl runs out, a new standard must be prepared.

Method Test. The precision, reproducibility of the method was tested by preparing standards as described above and by using varying amounts of 0.125 mg./ml. $SO_4^{=}$ standard prepared by dissolving 515.3 mg. Ammonium Sulfate in 1 liter of distilled water. The results of this measurement are recorded in Table I. The results and the Least Squares Fit are shown in figures I and II. The Standard Deviation as estimated by the Regression analysis is .0117. Therefore the absorbance value at 0 mg. could be 0 and the Equation reduces to comply with Beer's Law.

After finding that the method complies to Beer's Law if the lack of fit and random error are considered as residual error, it was necessary to establish if the fitting of the curve was a significant factor of the error. In order to test the hypothesis, duplicates were run of all the solutions used earlier. By fitting a curve to this data and performing a regression analysis it was found that the lack of fit was not a significant factor in the combined error at greater than 99.5 % probability (see Table II and figures III and IV). Therefore the model is shown of the absorbance on SO_4 concentration.

The relative standard deviation calculated on this data was 2.01 %. This indicates that the method has a precision of about

2 % (2.26 % on first sample data). Andersen states that the method has an accuracy of 2 %. Since he offers no substantiation of this, I assume he means reproducibility. Assuming this is the case, the above experimentation seems to confirm his findings.

Application to Blood Plasma. After obtaining results similar to Andersen's, attempts were made to measure blood plasma sulfate by this method. A plasma sample was prepared using 18 ml. of plasma and 12 ml. of 24 g./l. uranium acetate to remove the proteins and phosphate. The solution was centrifuged for 10 minutes at 3,000 revolutions per minute. The supernatant was pipetted off and filtered. 10 ml. of this supernatant was prepared as described in the sample procedure. Sources of the plasma sample believed that the sample contained approximately $10^{-3} M SO_4^{=}$. Therefore calculations were made of the sample prepared:

$$10^{-3} M (18 \text{ ml.}) = X (30 \text{ ml.})$$

$$X = 10^{-3} \left(\frac{18}{30}\right) = .6 \times 10^{-3} M$$

i.e. $.6 \times 10^{-3} M = .6 \times 10^{-3} \text{ mole/liter} = 57.6 \times 10^{-3} \text{ gm./l.}$

therefore .0576 mg./ml. and for a 10 ml. sample, .576 mg. $SO_4^{=}$

The measurements made on the solutions are recorded in Table III. No adsorption was observed in the plasma sample. Three conclusions could be drawn from this result: 1) The blood sample did not actually contain as much as $10^{-3} M$ Sulfate, or 2) The uranium acetate procedure removes the sulfate, or 3) This analysis method will not work on blood plasma.

Spiked Plasma Analysis. In order to insure that the sample contained a detectable amount of sulfate, 30 ml. of plasma was

spiked with 20 μ l. of 3N ammonium sulfate (prepared by dissolving 39.6438 gm. of dried ammonium sulfate in 100 ml. of distilled water). To this 20 ml. of 24 gm./l. uranium acetate was added. The solution was allowed 10 minutes to completely precipitate and was then centrifuged for 10 minutes at 3,000 rpm. The supernatant was removed and filtered. Two 10 ml. samples were taken and prepared as described under Sample Preparation. Each sample and standard was measured in duplicate. The results are recorded in Table IV and the analysis of the data in figures V and VI. The calculation of the sulfate added shows that each sample should contain 1.15 mg. of Sulfate:

$$3N \text{ SO}_4^{2-} \text{-----} \rightarrow 3 \text{ moles/l. (96.0616gm./mole)} = 288.185 \text{ gm./l.}$$

or .288185 mg./ μ l.

$$\frac{20 \mu\text{l. (.288185 mg./}\mu\text{l.)}}{30 \text{ ml.}} = .192123 \text{ mg. SO}_4^{2-}/\text{ml.}$$

$$\frac{30 \text{ ml. (.192123 mg./ml.)}}{50 \text{ ml.}} = .115274 \text{ mg. SO}_4^{2-}/\text{ml.}$$

$$\text{analyzing 10 ml.-----} \rightarrow 10 \text{ ml. (.115274 mg.SO}_4^{2-}/\text{ml.} = 1.15 \text{ mg.}$$

As indicated in figures V and VI, the average absorbance of the samples is equivalent to .368 mg. SO_4^{2-} which is very much less than the added amount of sulfate. These results would indicate that the problem results from the uranium acetate treatment or the failure of the analysis method since there was at least 1.15 mg. of sulfate in the sample. As a final attempt to salvage the method, samples were prepared by spiking the plasma samples after the uranium acetate treatment to test the remote possibility that the uranium acetate was removing the sulfate. Two 10 ml. samples were

obtained by adding 4 μ l. of 3N Ammonium Sulfate to 10 ml. of the filtered supernatant of 30 ml. of plasma treated with 20 ml. of 24 gm./l. Uranium Acetate. The results of the duplicate runs made on these and the standards are given in Table V and the analysis of the data in figures VII and VIII. The absorbance is equivalent to .425 mg. of sulfate which is far below the added amount 1.15 mg. sulfate:

$$4 \mu\text{l.} (.2881858 \text{ mg./}\mu\text{l.}) = 1.15 \text{ mg. SO}_4^{--}$$

The result shows conclusively that the benzidine precipitation method does not work in the plasma matrix. The plasma solution must make the benzidine more soluble. There seems to be some slight difference caused by the uranium acetate treatment but further investigation of this will be necessary before any conclusions can be drawn.

Conclusion. The results of the experimentation are self-explanatory. By using random measurement, it has been possible to analysis the data by regression method and obtain a relative standard deviation of about 2 %/o. This agrees well with Andersen's conclusions. The plasma tests reveal that the method is not accurate for measurements of sulfate in plasma. This is probably the result of a higher solubility of benzidine sulfate in the complex plasma solution. The test could be in error due to the presence of uranium acetate in the solution but the data would indicate that the uranium acetate is precipitating little, if any, sulfate. Since the benzidine does not work on the micro level no attempt was made to test the ultramicro method, since the same interferences should be involved. A possible method to try next would be the use

of CAD rather than benzidine as the precipitating agent since its sulfate salt is much more insoluble-but a detection method will need to be devised. A possibility is the detection of excess CAD by UV Spectrophotometry. If this method does not work, it would seem that the gas methods are the only accurate methods that could be used.

T A B L E S

TABLE I
TEST RUN No. 1

	MG SO ₄	<u>ABSORBANCE</u>
***	.125	.165
	.375	.223
	.500	.308
	.625	.371
	1.00	.585
	1.25	.725
	1.50	.900

***INCORRECT PROCEDURE FOLLOWED

FIGURE I

```

INPUT X, Y? .375, .223
INPUT X, Y? .500, .308
INPUT X, Y? .625, .371
INPUT X, Y? 1.00, .585
INPUT X, Y? 1.25, .725
INPUT X, Y? 1.50, .900
INPUT X, Y? 1E38, 1E38

```

```

SUM X = 5.25
SUM X^2 = 5.59375
SUM Y = 3.112
SUM Y^2 = 1.960084
SUM X*Y = 3.31075
THE B MATRIX IS:

```

```

6          .875          .5186667
5.25      1             .58775

```

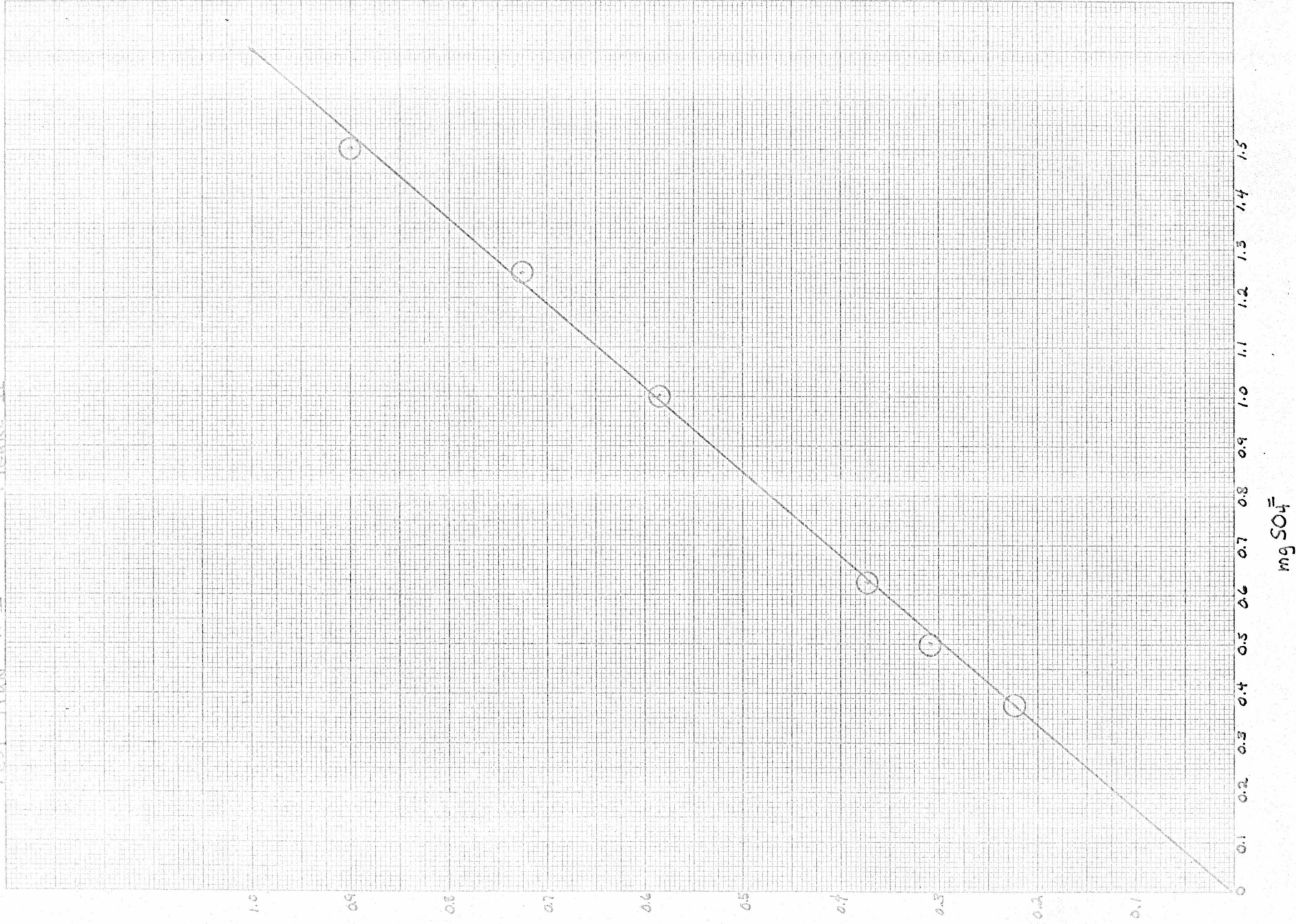
Y = 4.385397E-3 / .58775 X

RAT	SS	DF	MS	F
TOTAL	1.960084	6		
BO	1.614091	1	1.614091	
B1	.3454501	1	.3458204E-4	
SUBTOTAL	5.432814E-4	4	1.358204E-4	

S = .0117

RSD = .0177/.5187 = .0226 (100) = 2.26 per cent

TEST RUN #1 FIGURE II



FORBES

10 X 10 TO THE CENTIMETER 46 1510
K&M
18 X 25 CM.
KEUFFEL & ESSER CO.
PHILADELPHIA

TABLE II
Test Run no. 2

<u>mg. SO₄</u>	<u>ABSORBANCE</u>
.500	.307
1.00	.575
.625	.370
1.50	.900
.375	.220
1.25	.725
.500	.296
1.00	.585
.375	.224
1.500	.880
1.25	.750
.125	.074
.125	.079
.625	.375

TABLE III
Plasma Test

<u>mg. SO₄</u>	<u>ABSORBANCE</u>
.500	.289
1.00	.575
1.50	.900
SAMPLE	.000

FIGURE III

INPUT X,Y? .500,.307
 INPUT X,Y? 1.00,.575
 INPUT X,Y? .625,.370
 INPUT X,Y? 1.50,.900
 INPUT X,Y? .375,.220
 INPUT X,Y? 1.25,.725
 INPUT X,Y? .500,.296
 INPUT X,Y? 1.00,.585
 INPUT X,Y? .375,.224
 INPUT X,Y? 1.50,.880
 INPUT X,Y? 1.25,.750
 INPUT X,Y? .125,.074
 INPUT X,Y? .125,.079
 INPUT X,Y? .625,.375
 INPUT X,Y? 1E38,1E38

SUM X = 10.75
 SUM X² = 11.21875
 SUM Y = 6.36
 SUM Y² = 3.915058
 SUM X*Y = 6.6265
 THE B MATRIX IS:

14	.7678571	.4542857
10.75	2.964286	.5879759

$Y = 2.804216E-3 \cdot .5879759 X$

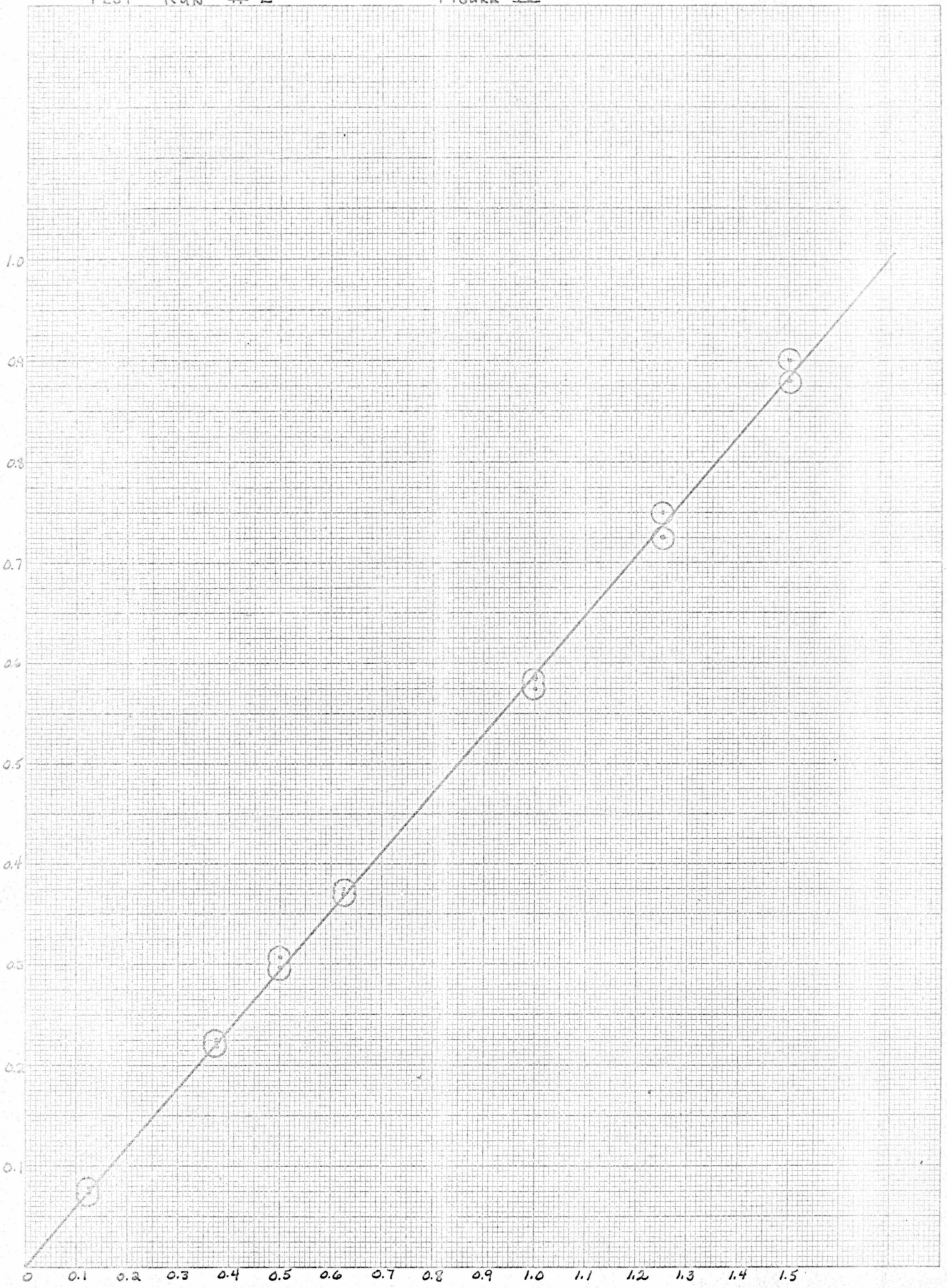
RAT	SS	DF	MS	F
TOTAL	3.915058	14		
B0	2.889257	1	2.889257	30736.82***
B1	1.0248	1	1.0248	10902.12***
SUBTOTAL	1.000941E-3	12	8.341173E-5	
ERROR	.656E-4	7	9.4E-5	
LACK OF FIT	.344941E-4	5	6.9E-5	.734 N.S.

$S = .00914$

$RSD = (.00914/.4542857) * 100 = 2.01 \text{ per cent}$

TEST RUN # 2

FIGURE IV



11 x 10 x 10 TO THE CENTIMETER 46 1510
1.9 x 2.5 CM.
REUFFEL & ESSER CO.

mg SO₄⁼

SPIKED SAMPLE RUNS

TABLE IV

<u>mg. SO₄</u>	<u>ABSORBANCE</u>
.500	.296
1.50	.90
SAMPLE 1	.210
.500	.258
SAMPLE 2	.200
1.00	.580
1.00	.570
SAMPLE 2	.19
SAMPLE 1	.220
1.50	.890

TABLE V

<u>mg. SO₄</u>	<u>ABSORBANCE</u>
1.50	.900
SAMPLE 2	.235
.500	.290
1.50	.890
SAMPLE 1	.232
SAMPLE 2	.245
1.00	.565
1.00	.576
.500	.280
SAMPLE 1	.220

FIGURE V

INPUT X,Y? .500,.296
 INPUT X,Y? 1.50,.900
 INPUT X,Y? .500,.285
 INPUT X,Y? 1.00,.580
 INPUT X,Y? 1.50,.890
 INPUT X,Y? 1E38,1E38

SUM X = 6
 SUM X² = 7
 SUM Y = 3.521
 SUM Y² = 2.432241
 SUM X*Y = 4.1255
 THE B MATRIX IS:

6	1	.5868333
6	1	.6045001

Y = -.01766673 / .6045001 X

RAT	SS	DF	MS	F
TOTAL	2.432241	6		
BO	2.06624	1	2.06624	38621.31***
B1	.3654203	1	.3654203	6830.286***
SUBTOTAL	5.805194E-4	4	1.451299E-4	
ERROR	1.605E-4	3	5.35E-5	
LACK OF FIT	4.200194E-4	1	4.200194E-4	7.85 N.S.

S = .0121

RSD = (.0121/.5868)* 100 = 2.06 per cent

SULFATE IN SAMPLES (CALCULATION BY EQUATION)

SAMPLE 1 .385 MG SULFATE
 SAMPLE 2 .352 MG SULFATE

AVERAGE .368 MG SULFATE IN THE PLASMA

SPIKED PLASMA SAMPLE FIGURE VI 1.15 mg SO_4^{2-} Spike

NEUFEL & DORRIP CO.

K.S. 10 X 10 TO THE 6 ANTIMETER 46 1510
MADE IN U.S.A.

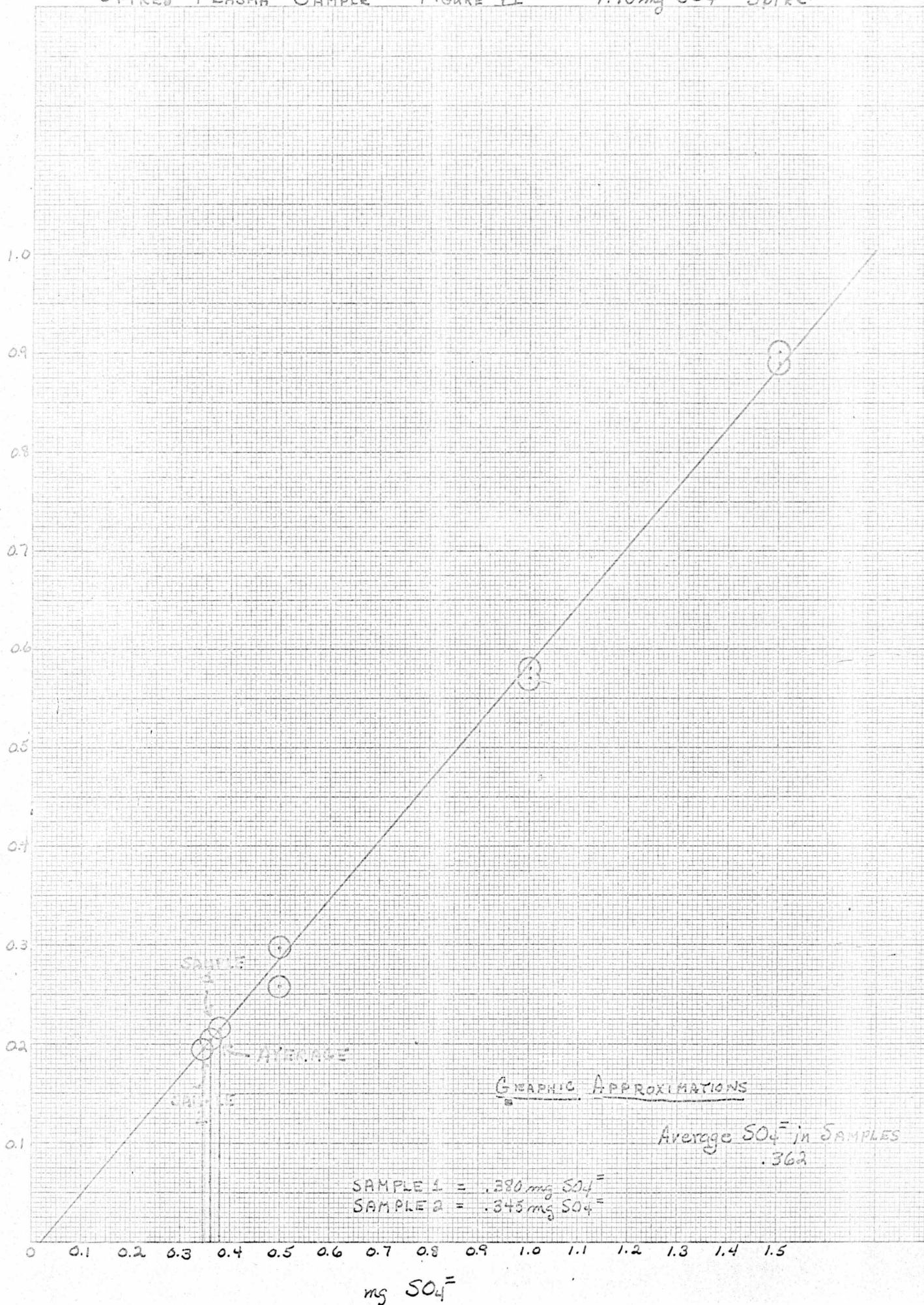


FIGURE VII

INPUT X,Y? 1.50,.900
 INPUT X,Y? .500,.290
 INPUT X,Y? 1.50,.890
 INPUT X,Y? 1.00,.565
 INPUT X,Y? 1.00,.576
 INPUT X,Y? .500,.280
 INPUT X,Y? 1E38,1E38

SUM X = 6
 SUM X² = 7
 SUM Y = 3.501
 SUM Y² = 2.415601
 SUM X*Y = 4.111

THE B MATRIX IS:

6	1	.5835
6	1	.61

$Y = -.02650002 + .61 X$

RAT	SS	DF	MS	F
TOTAL	2.415601	6		
B0	2.042834	1	2.042834	38183.81***
B1	.3721	1	.3721	6955.14***
SUBTOTAL	6.674826E-4	4	1.668707E-4	
ERROR	1.605	3	5.35E-5	
LACK OF FIT	5.069826E-4	1	5.069826E-4	9.47631 N.S.

$S = .0129$

$RSD = (.0129/.5835) * 100 = 2.21 \text{ per cent}$

SULFATE IN SAMPLES (CALCULATION BY EQUATION)

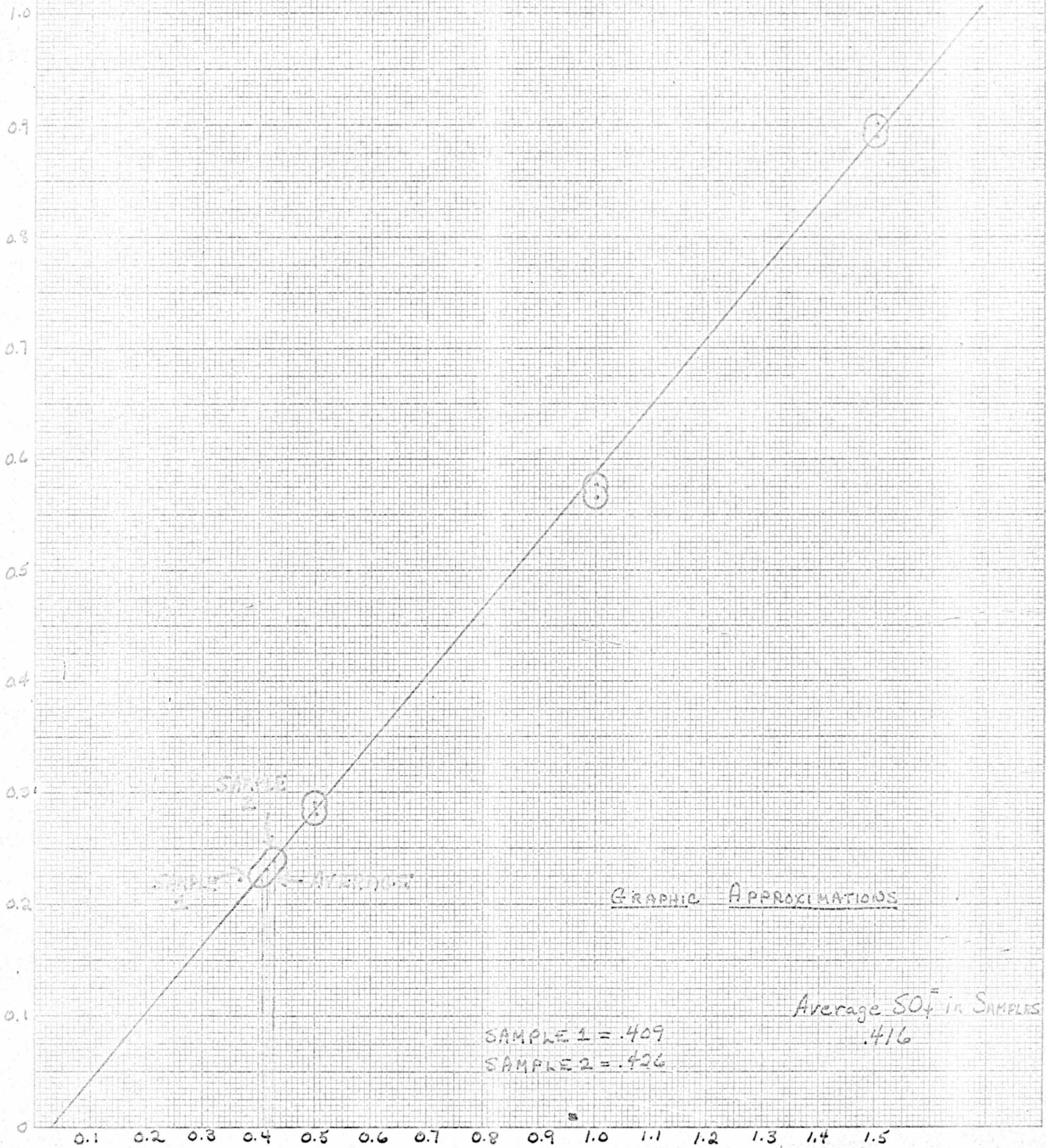
SAMPLE 1 .415 MG SULFATE
 SAMPLE 2 .437 MG SULFATE

AVERAGE .425 MG SULFATE IN THE PLASMA

SPIKED SAMPLE OF PLASMA (PHOSPHATE REMOVED FIRST) FIGURE VIII
 1.15 mg $SO_4^{=}$ Spike

MCP 277000000

10 X 10 TO THE CENTIMETER 4000
 MADE IN U.S.A.
 KUFFEL & ESSER CO.



GRAPHIC APPROXIMATIONS

Average $SO_4^{=}$ in SAMPLES
 .416

SAMPLE 1 = .409
 SAMPLE 2 = .426

mg $SO_4^{=}$

REFERENCES

1. M.A. Pleva, Washington and Lee Chemistry Department, personal conversation.
2. E. Miller, C.J. Hlad, S. Levine, J.H. Holmes, and H. Elrick, Jr. Lab. Clins. Med. 58, 656-66.
3. CA 64: 14962b
4. J.B. Davis, and F. Lindstrom, Anal. Chem. 44 (3), 524-32 (1972).
5. R. Belcher, A.J. Nuttent, and W.I. Stephen, J. Chem. Soc., 1953, 1334.
6. R. Belcher, and D. Gibbons, J. Chem. Soc. 1952, 4216.
7. R.L. Coleman, W.D. Shults, M.T. Kelly and J.A. Dean, Anal. Chem. 44 (6), 1031-34 (1972).
8. L. Andersen, Acta. Chem. Scand. 7(4), 689-692 (1953).
9. E. Miller, et. al., J. Lab. Clin. Med. 58, 656-66.

A P P E N D I X
PROGRAM FOR REGRESSION ANALYSIS

```

1 A=0
2 B=0
3 C=0
4 D=0
5 E=0
6 F=0
9 PRI
10 PRI "INPUT X,Y";
20 INP X,Y
30 IF X =1E38 THEN 100
40 A=A/X
50 B=B/X^2
60 C=C/Y
70 D=D/Y^2
80 E=E/X*Y
90 GOT 10
100 PRI
110 PRI "SUM X =";A
120 PRI "SUM X^2 =";B
130 PRI "SUM Y =";C
140 PRI "SUM Y^2 =";D
150 PRI "SUM X*Y =";E
200 S(1) =F
210 S(2) =A/F
220 S(3) =C/F
230 T(1) =A
240 T(2) =B-S(2)*A
250 T(3) =E-S(3)*A
260 T(3) = T(3)/T(2)
270 M=T(3)
280 O=S(3)-S(2)*T(3)
290 PRI "THE B MATRIX IS:"
300 PRI S(1); TAB (10); S(20); S(3)
310 PRI T(1);TAB (10);T(2);TAB(20);T(3)
320 PRI
330 PRI "Y=";O;" /";M;" X"
350 I=S(1)*(S(3)^2)
360 J=T(2)*T(3)^2
365 PRI
370 PRI "RAT";TAB(15);"SS";TAB(30);"DF";TAB (45);"MS";TAB (60);"F"
380 PRI "TOTAL";TAB (15);D;TAB (30);F
390 PRI "BO";TAB (15);I;TAB (31); "I";TAB (45); I
400 PRI "B1";TAB (15);J; TAB(31); "I"; TAB (45); J
410 PRI
420 L=D-(I/J)
422 K=F-2
425 W=L/K
430 PRI "SUBTOTAL"; TAB (15); L;TAB (30); K;TAB (45);W
440 PRI
444 PRI
445 PRI
500 END

```